Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein

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Porcine circovirus type 2 (PCV2), a single-stranded DNA virus associated with post-weaning multisystemic wasting syndrome of swine, has two potential open reading frames, ORF1 and ORF2, greater than 600 nucleotides in length. ORF1 is predicted to encode a replication-associated protein (Rep) essential for replication of viral DNA, while ORF2 contains a conserved basic amino acid sequence at the N terminus resembling that of the major structural protein of chicken anaemia virus. Thus far, the structural protein(s) of PCV2 have not been identified. In this study, a viral structural protein of 30 kDa was identified in purified PCV2 particles. ORF2 of PCV2 was cloned into a baculovirus expression vector and the gene product was expressed in insect cells. The expressed ORF2 gene product had a molecular mass of 30 kDa, similar to that detected in purified virus particles. The recombinant ORF2 protein self-assembled to form capsid-like particles when viewed by electron microscopy. Antibodies against the ORF2 protein were detected in samples of sera obtained from pigs as early as 3 weeks after experimental infection with PCV2. These results show that the major structural protein of PCV2 is encoded by ORF2 and has a molecular mass of 30 kDa.

Introduction

Porcine circovirus (PCV) is a member of the family Circoviridae (Lukert et al., 1995). Other members include psittacine beak and feather disease virus (Ritchie et al., 1989) and chicken anaemia virus (Todd et al., 1990). PCV was first identified by Tischer et al. (1974) as a contaminant of PK-15 cells. The virus is an isometric particle with a diameter of 17 nm and contains covalently closed circular single-stranded DNA of about 1.76 kb (Tischer et al., 1982). Experimental animal studies suggest that PCV from the PK-15 cell line is nonpathogenic for swine (Tischer et al., 1986; Allan et al., 1995).

Recently, PCV was found associated with the newly emerged post-weaning multisystemic wasting syndrome (PMWS) of swine (Allan et al., 1998; Ellis et al., 1998; Meehan et al., 1998; Morozov et al., 1998). Manifestations of PMWS include unthriftiness, respiratory distress and jaundice (Harding, 1997). Frequently encountered microscopic lesions are interstitial pneumonia, lymphoid depletion and hepatitis (Clark et al., 1997). Monoclonal antibodies raised against PCV in the PK-15 cell line have been used to differentiate that virus from PCV associated with PMWS (Allan et al., 1998). Genetic analysis clearly indicates that two genotypes of PCV exist (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). PCV in the PK-15 cells represents type 1 (PCV1), and PCV associated with PMWS segregates into a second virus genotype, PCV2 (Meehan et al., 1998).

To date, structural protein(s) of PCV2 and the open reading frame(s) (ORF) that code for viral structural protein(s) have not been identified. In this study, we identified a protein of 30 kDa as a viral structural protein which is encoded by ORF2 of PCV2. This protein independently forms viral capsid-like structures when expressed in insect cells from recombinant baculovirus.

Methods

*Cells.* Porcine kidney 15 (PK-15) cells free of PCV were obtained from K. M. Lager at the National Animal Disease Center, Ames, Iowa, USA. Cells were propagated in minimum essential medium (Gibco BRL)
with 5% foetal bovine serum (FBS) (Gibco BRL) and maintained at 37 °C in 5% CO₂. Spodoptera frugiperda (Sf9) cells were purchased from Invitrogen and were cultured in Grace’s insect media (Gibco BRL) supplemented with 10% FBS (Gibco BRL) at 27 °C.

**Viruses.** To produce purified virus, PCV2 strain ISU31 (Morozov et al., 1998) was propagated in the PK-15 cell line as described by Tischer et al. (1986). The infected cells were frozen and thawed three times, clarified at 27000 g for 30 min and pelleted at 270000 g for 6 h. The pellet was resuspended in NET buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA and 10 mM MgSO₄) and centrifuged in a solution of 37% CsCl (w/v) in water to equilibrium for 48 h at 270000 g. A readily visible opaque band was collected by aspiration, washed and dissolved in NET buffer.

To make PCV2 stock, purified PCV2 strain ISU31 was inoculated onto a semi-confluent PCV-free PK-15 cell line according to the method of Tischer et al. (1986). Briefly, the infected cells were treated with 300 mM N-glucosamine (Sigma) for 30 min at 37 °C at 4 h post-inoculation (p.i.). When the cells were confluent, they were trypsinized and passaged. The cells were treated with N-glucosamine (300 mM) 1 day after subculture. When 80% of the cells were showing cytopathic effect, the infected cells were frozen and thawed three times and the resulting suspension of cell debris and membrane was clarified by centrifugation. The viruses in the supernatant were pelleted at 270000 g for 6 h. The virus pellet was treated with an equal volume of Freon (Fisher Scientific) and filtered through a 0.2 μm filter.

Wild-type (wt) Autographa californica nuclear polyhedrosis virus (AcMNPV) and recombinant AcMNPV expressing ORF2 (AcMNPV.ORF2) stocks were prepared as previously described (O’Reilly et al., 1992). To prepare working stock of wild-type and recombinant baculoviruses, wt AcMNPV and AcMNPV.ORF2 were propagated in Sf9 monolayer cultures for 5 days. The infected cells and supernatant were frozen and thawed three times and clarified by centrifugation at 11000 g for 10 min.

**Molecular cloning and expression of viral genes.** A Bac-to-Bac baculovirus expression system (Gibco BRL) was used for expression of the ORF2 gene. Viral DNA was isolated from PK-15 cells infected with the ISU31 strain of PCV2. ORF2 of PCV2 strain ISU31 was amplified by PCR using primers 5' AGT GCT CGA GGG ATC CAT GAC GTA TCC AGG GAG GCG 3’ and 5’ GAG CAG ATC TTT AGG GTT TAA GTG GGG GGT CTT TAA G 3’ (sequence specific for PCV2 ORF2 is in bold type; sequence engineered to create cloning sites is in roman type). The ORF2 fragment was cloned into XhoI and BglII sites of plasmid pKSI” (Stratagene). The integrity of the base sequence of ORF2 in pKSI” was verified by sequence analysis. To produce the donor recombinant plasmid (pPSP.PCV.ORF2) for derivation of recombinant baculovirus, the ORF2 gene was subcloned into pFastbac (Gibco BRL) at BamHI and SpeI restriction sites. Recombinant baculovirus carrying the ORF2 gene was constructed according to the manufacturer’s instructions (Bac-to-Bac baculovirus expression system, Gibco BRL). Briefly, E. coli DH10Bac (Gibco BRL) containing baculovirus shuttle vector (bacmid) and helper vector was transformed with recombinant plasmid pPSP.PCV.ORF2. Within E. coli DH10Bac, the ORF2 gene was transposed into the bacmid. The colonies of E. coli containing recombinant bacmid were collected by blue/white selection. The recombinant bacmid DNA was isolated, purified and transfected into Sf9 cells to yield AcMNPV carrying the PCV2 ORF2 gene, referred to as AcMNPV.ORF2, under the control of the polyhedrin promoter. Primary stock of AcMNPV.ORF2 was harvested at 72 h post-transfection. Expression of the ORF2 gene of PCV2 was confirmed by indirect immunofluorescent assay using hyperimmune serum raised against PCV2 in rabbits (Sorden et al., 1999).

**Purification of recombinant ORF2 expression product.** Purified ORF2 expression protein was obtained from lysates of ORF2 gene product. Sf9 cells infected with AcMNPV.ORF2 were lysed at 72 h p.i. according to the method of Wong et al. (1994) and purified by CsCl gradient centrifugation as described for PCV2. Briefly, AcMNPV.ORF2-infected Sf9 cells were lysed in buffer containing 50 mM sodium borate, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 5% β-mercaptoethanol. The lysates were diluted in PBS, laid on top of 40% sucrose in PBS and centrifuged at 270000 g for 6 h. The pellet was resuspended in NET buffer and centrifuged to equilibrium at 37% CsCl in water at 270000 g for 48 h. The opaque band in the middle of the tube was collected and dialysed in Tris buffer (50 mM Tris and 150 mM NaCl, pH 7.2) for 24 h with three changes of the buffer. Sf9 cells and wt AcMNPV-infected Sf9 cells were lysed and purified by a method similar to that used for AcMNPV.ORF2-infected Sf9 cells.

**Cell lysates.** The PCV2 purified by CsCl gradient centrifugation was lysed with an equal volume of Laemml sample buffer (Bio-Rad) and boiled for 5 min. To prepare cell lysates from PCV2-infected cells, 25 cm² flasks of semi-confluent PCV-free PK-15 cells were infected with 1 ml Laemml sample buffer (Bio-Rad). The cells were incubated with the sample buffer for 2 min on ice before they were scraped, transferred to a microfuge tube and boiled for 5 min.

To prepare lysates from baculovirus-infected cells, wt AcMNPV and AcMNPV.ORF2 were inoculated onto monolayers of Sf9 cells at an m.o.i. of 5. Mock-infected Sf9 cells were used to prepare control cell lysates. The cells were lysed at 72 h p.i. as described previously. Recombinant ORF2 expression protein lysate was prepared from the purified ORF2 protein, containing virus-like particles, which was dissolved in an equal volume of Laemml sample buffer (Bio-Rad), boiled for 5 min and used for electrophoresis.

**Preparation of anti-PCV sera.** Rabbit anti-PCV2 hyperimmune serum was prepared as described elsewhere (Sorden et al., 1999). The gradient-purified PCV2 was diluted in 0.85% NaCl to a concentration of 1 mg/ml and mixed with adjuvant MLP + TPM + CWS (Sigma). The rabbits were inoculated with PCV2-adjuvant emulsion. Immunizations were repeated a total of three times at 3-week intervals. The serum was
collected 2 weeks after the final immunization and tested for the presence of antibodies to PCV2 by indirect immunofluorescent assay. The serum was absorbed with PK-15 cells or SF9 cells as described by Harlow & Lane (1988).

Samples of swine serum were obtained from a pig experimentally inoculated with strain 35358 of PCV2 at 0, 7, 14, 21, 28, 36, 42 and 49 days p.i.

**Western blot.** SDS–PAGE was done according to the method of Laemmli (1970) using a 1 mm thick 15% slab gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Bio-Rad) using a mini Trans-Blot transfer cell (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, 20%, v/v, methanol) at 100 V for 90 min. Immunoblots were performed as described by Harlow & Lane (1988) and Zhang et al. (1998) with slight modification. The nitrocellulose membrane was soaked in 0.5% blocking solution (Roche) before incubation with rabbit anti-PCV2 serum (1:3000) or swine anti-PCV2 serum (1:100) overnight at 4 °C. The blots were reacted with peroxidase-labelled anti-rabbit or anti-swine IgG (KPL) for 1 h at room temperature. The membranes were washed five times with Tris buffer saline (50 mM Tris pH 7.5, 200 mM NaCl) containing 0.1% Tween 20 between each incubation. After equilibration in 0.15 M Tris pH 9.5, bound antibodies were detected with 3,3’5,5’-tetramethylbenzidine (TMB) substrate peroxidase solution (KPL).

**Negative staining electron microscopy and immunoelectron microscopy.** Purified PCV2 and purified ORF2 expression product from the CsCl gradient centrifugation were allowed to absorb onto carbon-coated copper grids by agar diffusion for 30 min. The grids were dried and negatively stained with 3% phosphotungstic acid for 5 min. Then, the grids were absorbed with 1% Tween 20 between each incubation. After equilibration in 0.15 M Tris pH 9.5, bound antibodies were detected with 3,3’5,5’-tetramethylbenzidine (TMB) substrate peroxidase solution (KPL).

**Results**

**Immunoblot analysis showing 30 kDa protein as a major structural protein**

A single protein of approximately 30 kDa was detected when SDS–PAGE followed by immunoblotting (Fig. 1). The proteins from PK-15 cells, PCV2-infected PK-15 cells, gradient-purified PCV2 particles, AcMNPV.ORF2-infected SF9 cells, wt AcMNPV-infected SF9 cells and non-infected SF9 cells were transferred to a nitrocellulose membrane and incubated with rabbit anti-PCV2 hyperimmune serum overnight at 4 °C. The nitrocellulose membrane was subsequently incubated with peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature before reacting with TMB substrate. A 30 kDa protein was detected in PCV2-infected PK-15 cell lysates and in gradient-purified PCV2 particles. A protein of similar size was also detected in the lysates of AcMNPV.ORF2-infected SF9 cells but not in the lysates of wt AcMNPV-infected and non-infected SF9 cells (Fig. 1). A Coomassie blue-stained gel of electrophoresed lysates of AcMNPV.ORF2-infected SF9 cells showed high level expression of a protein of 30 kDa (Fig. 2). Isopycnic gradient purification of lysates of AcMNPV.ORF2-infected SF9 cells or PCV2-infected PK-15 cells resulted in the formation of a distinct band in the middle of the tube. The band was collected and had a refractive index of approximately 1.36. The protein band was absent in the tubes containing mock-infected SF9 cells and wt AcMNPV-infected cell lysates, when observed under scatter light. When the protein band was electrophoresed on SDS–polyacrylamide gel and stained with Coomassie blue, the results showed that the recombinant ORF2 protein was partially pure (Fig. 2, lane
2); however, the 30 kDa protein was the most abundant protein. Further analysis of the band by Western blot revealed that a protein of 30 kDa reacted specifically with rabbit anti-PCV2 hyperimmune serum (Fig. 3). A protein of 30 kDa was not detected in the lanes containing lysates from mock-infected Sf9 cells or wt AcMNPV-infected Sf9 cells.

Recombinant ORF2 product assembles into virus-like particles

When the recombinant expression product from ORF2 in insect cells was partially purified by gradient centrifugation, numerous virus-like particles were detected by negative staining electron microscopy (Fig. 4). The self-assembled virus-like particles appeared to be of lower density than that of intact PCV2 particles (Fig. 4). The self-assembled particles were of similar morphology to the PCV2 virion and some self-assembled virus-like particles had darkly stained centres that made them appear to be empty capsids. Both PCV2 particles and self-assembled particles were approximately 17 nm in diameter.

Immunoelectron microscopic results confirmed that the capsid-like particles were recombinant ORF2 protein that self-assembled to form capsid-like structures. The recombinant ORF2 particles clumped together with the serum containing antibody to PCV2 but not with preimmune serum (Fig. 4).

Detection of antibodies against ORF2 recombinant protein in swine serum

To determine the antigenic authenticity of the expression product from ORF2, samples of serum obtained from swine at
periodic intervals after experimental infection with PCV2 were
used for immunoblotting. Blots were prepared from lysates of
S9 cells infected with recombinant AcMNPV.ORF2 and
incubated with swine sera obtained at 0, 7, 14, 21, 28, 35, 42
and 49 days p.i. The results showed that antibodies against the
ORF2 expression product were detected as early as 21 days
after infection (Fig. 5). The intensity of the signal on the
Western blot indicated that the antibody titre increased in
serum over time after challenge exposure of pig with virus.

Discussion

In this study, we have identified a 30 kDa protein as a
major structural protein of PCV2. Identification of one major
structural protein is similar to the results obtained after analysis
of PCV1 (Tischer et al., 1982). However, the molecular mass of
the major structural protein of PCV1 was reported to be
36 kDa. Whether this discrepancy reflects a true difference in
molecular mass of the two PCV types or experimental variation
is not known. PCV1 and PCV2 are related antigenically and
have identical morphology (Allan et al., 1998; Ellis et al., 1998).
Additionally, both PCVs have two similar major ORFs, ORF1
and ORF2 (Meehan et al., 1997, 1998; Hamel et al., 1998;
Morozov et al., 1998). The predicted amino acid length of
proteins encoded by ORF2 of both PCV1 and PCV2 is 233
amino acids (Hamel et al., 1998). Although ORF2 of PCV1 and
of PCV2 was predicted to contain one glycosylation site
(Hamel et al., 1998), it is possible that it may encode proteins
with different numbers of sites for post-translation modification
such as phosphorylation, sulfation, methylation, acetylation
and hydroxylation that might account for the different sizes of
the proteins.

Both ORF1 and ORF2 have a theoretical coding capacity of
proteins of 28 kDa or larger (Hamel et al., 1998; Meehan et al.,
1998; Morozov et al., 1998). The predicted amino acid
sequence of ORF1 from either PCV1 or PCV2 revealed at least
three amino acid motifs found in the Rep protein which are
associated with rolling circle replication (Mankertz et al.,
1997; Meehan et al., 1997, 1998; Morozov et al., 1998).
Additionally, a plasmid containing ORF1 of PCV1 enhanced
replication of the PCV1 origin of replication, confirming that
ORF1 of PCV1 encodes the Rep protein (Mankertz et al.,
1998a). On the other hand, the predicted amino acid sequence
of ORF2 from either PCV1 or PCV2 contained a conserved
region of basic amino acids at the N-termini, similar to that
observed for the major structural protein of chicken anaemia
virus (Meehan et al., 1998). For PCV1, transcription analysis
indicated that the ORF2 transcript was the most abundant
(Mankertz et al., 1998b), corresponding to our finding of a high
level expression of the 30 kDa protein in PCV2-infected PK-15
cells. The predicted molecular mass of the translated product
of ORF2 is 28 kDa (Hamel et al., 1998; Meehan et al., 1998;
Morozov et al., 1998), which is close to the estimated 30 kDa
expression product of ORF2 reported here.

Our studies show that ORF2 encodes a 30 kDa protein that
is involved in viral capsid formation. Virus-like particles formed
by the expression product of ORF2 were similar to intact PCV
particles but some of the self-assembled virus-like particles
appeared empty. These findings are consistent with those
reported for several non-enveloped viruses, such as parvovirus
(Brown et al., 1991; Christensen et al., 1993), polyomavirus (An
et al., 1999), calcivirus (Geissler et al., 1999; Prasad et al., 1999)
and hepatitis E virus (Xing et al., 1999). Expression of the major
structural protein of each of the aforementioned viruses gave
rise to the formation of empty capsids (Brown et al., 1991; An
et al., 1999). Some of the ORF2 self-assembled particles
observed in our study were less ordered than those in the
purified PCV2 preparation. A similar finding was reported for
the mild truncated minor capsid protein of parvovirus B19
(VP1) expressed in baculovirus, and probably resulted from
improper assembly (Wong et al., 1994). The major capsid
protein (VP2) of parvovirus B19 is the truncated version at the
N terminus of its minor structural protein counterpart (VP1)
and both are transcribed from the same gene (Shade et al.,
1986; Ozawa & Young, 1987). In the case of PCV2, whether
this morphology results from the lack of minor structural
protein(s) or a requirement of DNA for perfect assembly needs
to be further investigated.

Antibodies raised against the ORF2 gene product could be
clearly detected as early as 21 days after infection. These
antibodies persisted for 49 days (the duration of experiment). These studies suggest a potential use of recombinant 30 kDa protein for the detection of PCV infection in pigs and as a vaccine.

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